RESEARCH REPORTS
Biological

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J Dent Res 81(8):531-535, 2002

Stem Cell Properties of Human Dental Pulp Stem Cells

ABSTRACT

In this study, we characterized the self-renewal capability, multi-lineage differentiation capacity, and clonogenic efficiency of human dental pulp stem cells (DPSCs). DPSCs were capable of forming ectopic dentin and associated pulp tissue *in vivo*. Stromal-like cells were reestablished in culture from primary DPSC transplants and re-transplanted into immunocompromised mice to generate a dentin-pulp-like tissue, demonstrating their self-renewal capability. DPSCs were also found to be capable of differentiating into adipocytes and neural-like cells. The odontogenic potential of 12 individual single-colony-derived DPSC strains was determined. Two-thirds of the single-colony-derived DPSC strains generated abundant ectopic dentin *in vivo*, while only a limited amount of dentin was detected in the remaining one-third. These results indicate that single-colony-derived DPSC strains differ from each other with respect to their rate of odontogenesis. Taken together, these results demonstrate that DPSCs possess stem-cell-like qualities, including self-renewal capability and multi-lineage differentiation.

KEY WORDS: stem cell, odontoblasts, dentin, *in vivo* transplantation.

Received December 27, 2001; Last revision April 26, 2002; Accepted June 5, 2002

A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org

INTRODUCTION

Stem cells are generally defined as clonogenic cells capable of both self-renewal and multi-lineage differentiation. Post-natal stem cells have been isolated from various tissues, including bone marrow, neural tissue, skin, retina, and dental epithelium (Harada *et al*., 1999; Fuchs and Segre, 2000; Bianco *et al*., 2001; Blau *et al*., 2001). Recently, we have identified a population of putative post-natal stem cells in human dental pulp, dental pulp stem cells (DPSCs). The most striking feature of DPSCs is their ability to regenerate a dentin-pulp-like complex that is composed of mineralized matrix with tubules lined with odontoblasts, and fibrous tissue containing blood vessels in an arrangement similar to the dentin-pulp complex found in normal human teeth (Gronthos *et al*., 2000).

Previous studies have demonstrated that, like osteoblasts, pulp cells express bone markers such as bone sialoprotein, alkaline phosphatase, type I collagen, and osteocalcin (Kuo *et al*., 1992; Tsukamoto *et al*., 1992; Nakashima *et al.*, 1994; Butler *et al*., 1997; Shiba *et al*., 1998; Buurma *et al*., 1999; Buchaille *et al*., 2000). Their differentiation is regulated by various potent regulators of bone formation, including members of the TGFβ superfamily and cytokines (Kettunen *et al*., 1998; Shiba *et al*., 1998; Onishi *et al*., 1999). The similarity of the gene expression profiles between DPSCs and precursors of osteoblasts, bone marrow stromal stem cells (BMSSCs), has recently been reported (Shi *et al*., 2001).

BMSSCs have been defined, by *in vitro* and *in vivo* studies, as pluripotential adult stem cells (Prockop, 1997; Bianco *et al*., 2001). They possess the capacity to differentiate into different kinds of cells such as osteoblasts, chondrocytes, adipocytes, muscle cells, and neural cells (Azizi *et al*., 1998; Fuchs and Segre, 2000; Bianco *et al*., 2001). In contrast, DPSCs have not yet been extensively studied in terms of their stem cell properties. Here, we demonstrate that human DPSCs represent a novel adult stem cell population that possesses the properties of high proliferative potential, the capacity of self-renewal, and multi-lineage differentiation.

MATERIALS & METHODS

Subjects and Cell Culture

Normal human third molars were collected from adults (19-29 yrs of age) at the Dental Clinic of the National Institute of Dental and Craniofacial Research under approved guidelines set by the National Institutes of Health Office of Human Subjects Research. For multi-colony- and single-colony-derived cell cultures, human DPSCs and BMSSCs were isolated and cultured as previously reported (Kuznetsov *et al*., 1997; Gronthos *et al*., 2000). For the culture of re-isolated DPSCs, three-month-old DSPC transplants were minced and then digested in a solution of 3 mg/mL collagenase type I

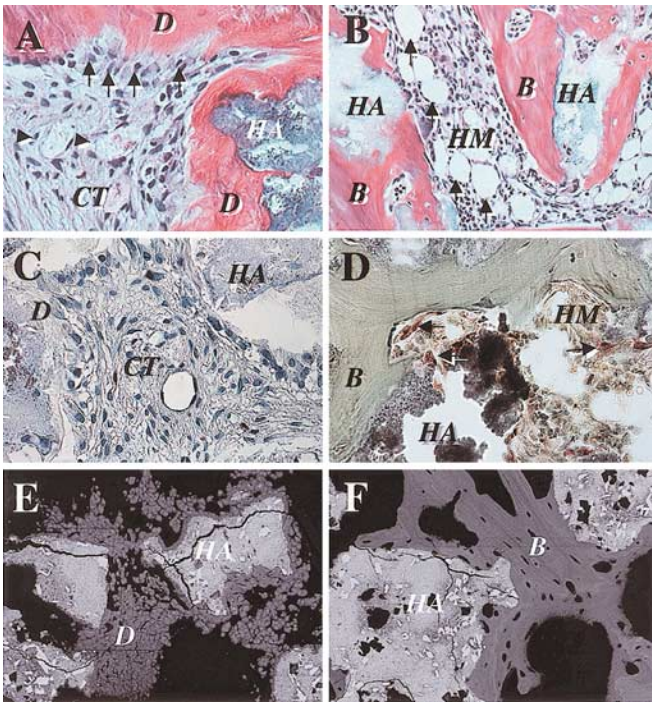

Figure 1. Characterization of DPSC transplant. **(A)** The dentin (*D*) generated in DSPC transplants was associated with connective tissue (*CT*), organized in a fashion similar to that of the tissue structure in dental pulp, containing odontoblasts (arrows) lining the surface of dentin (*D*), fibrous tissue, and blood vessels (triangles). **(B)** BMSSC transplants showed newly generated bone (*B*) surrounding hematopoietic marrow elements (*HM*) containing adipocytes (arrows). **(C)** DPSC transplants show negative staining for tartrate-resistant acid phosphatase (TRAP). **(D)** TRAP-positive osteoclasts were found in BMSSC transplants (arrows). **(E,F)** Back-scattered electron microscopy of DPSC and BMSSC transplants, respectively. A mineralized globular dentin-like structure (*D*) was found around the surfaces of HA/TCP (*HA*) (E) and mineralized bone lamellae (B) covered the surfaces of HA/TCP (*HA*) in BMSSC transplants (F).

and 4 mg/mL dispase for 1 hr at 37°C. For the induction of adipogenesis, a mixture including 0.5 mM isobutylmethylxanthine, 0.5 μM hydrocortisone, and 60 μM indomethacin was added to culture DPSCs for 5 wks (Gimble *et al*., 1995).

Transplantation

Approximately 4.0 x 10^6 DPSCs or BMSSCs (at 20-30 population doublings) were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc., Warsaw, IN, USA) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid, Harlan Sprague-Dawley, Indianapolis, IN, USA) as previously described (Krebsbach *et al*., 1997). These procedures were performed in accordance with specifications of an approved small-animal protocol (NIDCR #00-113).

Anti-human Dentin Sialoprotein Antibody and Immunohistochemistry

Antisera to a portion of the dentin sialoprotein (DSP) fragment of human dentin sialophosphoprotein (DSPP) was produced. Oligonucleotides were constructed to facilitate PCR amplification and subcloning of the Thr132-Asp373 domain of DSPP from a single exon, with human genomic DNA as a template. The PCR product was subcloned into pET-15b bacterial expression vector (NovaGen, Madison, WI, USA), and the induced fusion protein purified on a Ni^{2+} charged column. The purified product was injected 4 x (~ 0.25 mg each) into New Zealand white rabbits for antisera production. One rabbit (LF-151) produced a serum that showed, by Western analysis, good reactivity against human and bovine, but not murine, dentin extracts.

Unstained sections, deparaffinized with xylene and ethanol, were reacted with primary dentin sialoprotein antibody (1:100 dilution of LF-151). A Zymed broad-spectrum immunoperoxidase kit (Zymed Laboratories, South San Francisco, CA, USA) was used for staining, according to the manufacturer's protocol.

Histochemistry

The Accustain Trichrome Stain (GOMORI, Sigma HT-10-7, HT-10-9, and HT-10-5-16) and leukocyte acid phosphatase kit (Sigma #387-A) were used to stain paraffin-embedded sections for Trichrome and TRAP (Tartrate Resistant Acid Phosphatase), respectively, according to the manufacturer's protocols.

Back-scattered Electron Scanning Microscopy (BSE SEM)

Electron microscopy imaging was conducted on carbon-coated, polished block faces of poly-methyl-methacrylate (PMMA) embedded DPSC and BMSSC transplants for identification of the characteristics of the mineralized tissue phases. The samples were imaged in a Zeiss DSM962 digital scanning electron microscope operated at 20 or 30 kV in the back-scattered electron mode with the use of a KE (Toft, Cambs, UK) solid-state back-scatter electron detector.

RT-PCR

Total RNA was prepared from DPSCs, by means of the RNA STAT-60 (TEL-TEST Inc., Friendswood, TX, USA). First-strand cDNA synthesis was performed by means of a first-strand cDNA synthesis kit (GIBCO BRL, Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. The primer set for PCR included: PPARγ2 (sense, 5′-CTCCTATTGACCCAGAAAGC-3′, antisense, 5′-GTAGAGCTGAGTCTTCTCAG-3′, GenBank accession number XM_003059); LPL (sense, 5′-ATGGAG AGCAAAGCCCTGCTC-3′, antisense, 5′-GTTAGGTCCAGCT GGATCGAG-3′, GenBank accession number XM_044682); glial fibrillary acid protein (GFAP) (sense, 5′-CTGTTGCCAG AGATGGAGGTT-3′, antisense, 5′-TCATCGCTCAGGAGG TCCTT-3′, GenBank accession number XM_050159); nestin (sense, 5′-GGCAGCGTTGGAACAGAGGTTGGA-3′, antisense, 5′-CTCTAAACTGGAGTGGTCAGGGCT3′, GenBank accession number X65964); GAPDH (sense, 5′-AGCCGCATCTT CTTTTGCGTC-3′, antisense, 5′-TCATATTTGGCAGGTTTTTCT-3′, GenBank accession number M33197). The reactions were pre-incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA, USA) at 94°C for 2 min and then cycled 35 times at 94°C/(45 sec), 56°C/(45 sec), 72°C/(60 sec), followed by a final seven-minute extension at 72°C.

In situ Hybridization

Human-specific alu and mouse-specific pf1 sequences labeled with digoxigenin were used as probes for *in situ* hybridization as previously described (Gronthos *et al*., 2000). Primers for human alu (sense, 5′-TGGCTCACGCCTGTAATCC-3′, and antisense, 5′-TTTTTTGAGACGGAGTCTCGC-3′, GenBank accession number A0004024) and mouse pf1 (sense, 5′-CCGGGCAGTG GTGGCGCATGCCTTTAAATCCC-3′, and antisense, 5′-GTTTGGTTTTTGAGCAGGGTTCTCTGTGTAGC-3′, GenBank accession number X78319) were created.

Flow Cytometric Identification of Human and Mouse Cells

Adherent monolayers of stromal-like cells isolated from three-month-old DPSC transplants were digested with trypsin/EDTA to obtain single cell suspensions. CD-29 purified mouse anti-human or mouse-specific IgG (10 μg/mL) was then added directly to 2 x 10^5 cells for 1 hr on ice. The cells were then incubated with goat anti-mouse IgM conjugated to FITC (1/50 dilution, DAKO Corp., Carpinteria, CA, USA) for 45 min on ice. After being washed twice in PBS, the cells were analyzed with the use of a FACSCalibur flow cytometer. Positive expression was defined as the level of fluorescence greater than 99% of the corresponding isotype-matched control antibodies.

RESULTS

We demonstrated that the regenerated connective tissue formed by *ex vivo* expanded DPSCs was a dentin-pulp-like structure without an active hematopoietic marrow (Figs. 1A, 1C). Equivalent BMSSC transplants were composed of ectopic bone, undergoing normal turnover as demonstrated by the presence of osteoclasts at the regenerated bone surfaces, and active hematopoiesis and adipogenesis, none of which was present in the dentin-pulp complex of the DPSC transplants (Figs. 1B, 1D). In addition, back-scattered electron scanning microscopy showed that DPSCs formed a mineralized matrix with a globular, calcospheritic mineralization pattern similar to that of primary dentin (Fig. 1E), and distinct from that seen in the ectopic lamellar bone observed in BMSSC transplants (Fig. 1F).

To assess the self-renewal potential of DPSCs, we re-isolated stromal-like cells from three-month-old primary DPSC transplants. It was found that 85% and 15% of the cells were of human and mouse origin, respectively (Figs. 2A, 2B), based on FACS analysis with the use of either human- or mouse-specific

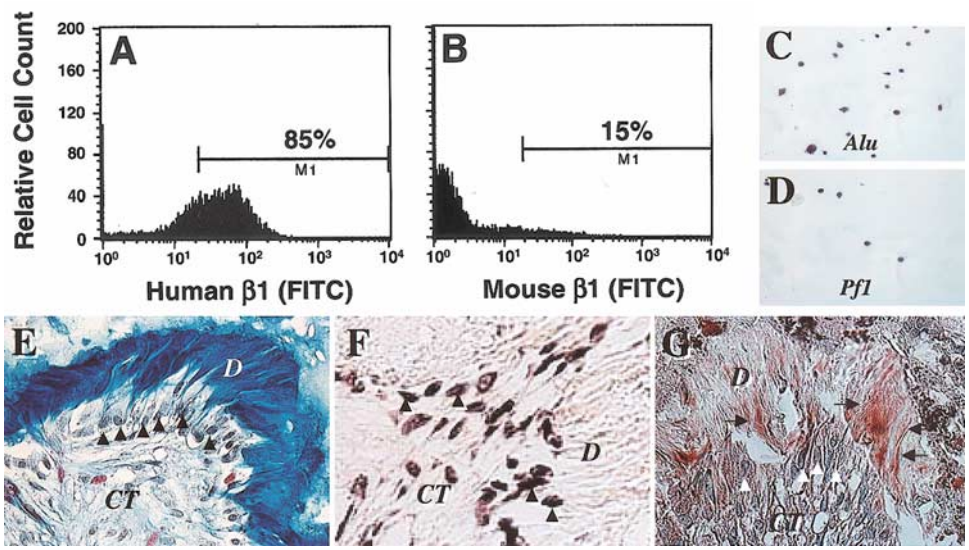

Figure 2. Isolation of stromal-like cells from DPSC transplants. Cells from DPSC transplants were isolated by fluorescence-activated cell sorting (FACS) as described in MATERIALS & METHODS. The majority of cells (85%) isolated from three-month DPSC transplants reacted with human-specific anti-CD29 monoclonal antibody **(A)** while the remaining cells (15%) reacted with the mouse-specific anti-CD29 antibody **(B)** using FACS. Similar results were obtained with human-specific alu **(C)** and mouse-specific pf1 **(D)** *in situ* hybridization, where the majority of cells were of human and far fewer cells of mouse. Trichrome staining **(E)**, human alu *in situ* hybridization **(F)**, and immunohistochemical staining **(G)** showed that these stromal-like cells, re-transplanted into immunocompromised mice, differentiated into alu-positive odontoblasts (triangles) that generated dentin (*D*) and linked to pulp-like connective tissue (*CT*). The newly generated dentin contained organized collagen fibers running perpendicular to the forming surface (blue color in E) and was positive for human DSP antibody staining (arrows in G).

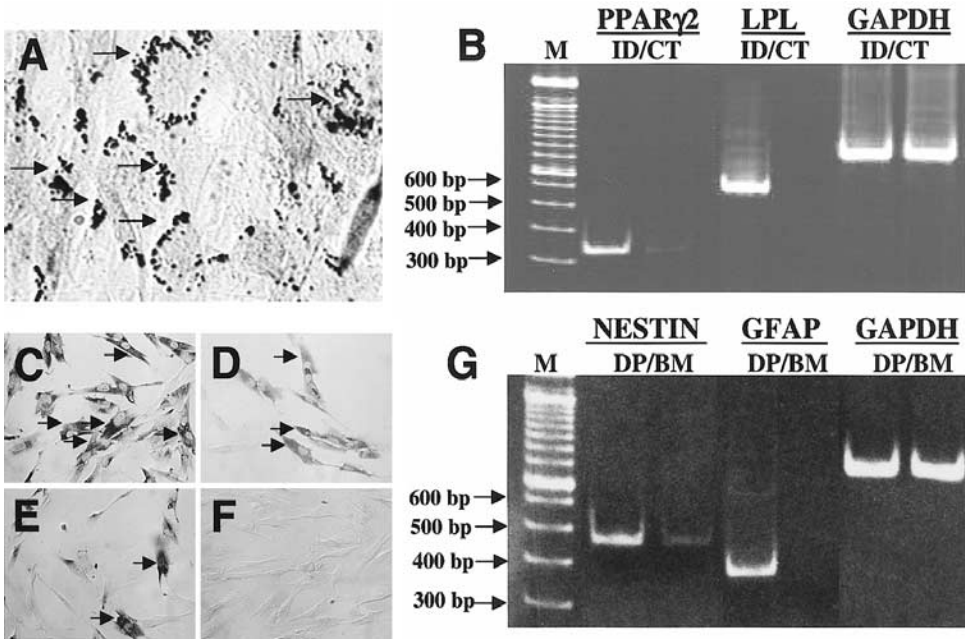

Figure 3. Adipogenic and neural differentiation of human DPSCs. In an adipogenic medium, DPSCs formed Oil red O-positive lipid clusters (arrows in **A**) and showed a significant up-regulation of PPARγ2 and lipoprotein lipase (LPL) in the induced group (ID) as compared with the control group (CT) by RT-PCR **(B)**. No measurable levels of lipoprotein lipase were detected in the control group (B). DPSCs were also immunostained for Nestin **(C)** and GFAP **(D)**, while BMSSCs were immunoreactive only for Nestin **(E)** and not for GFAP **(F)**. Similar results were obtained from RT-PCR **(G)**. DPSCs (DP) expressed both Nestin and GFAP at a high level when compared with the level of Nestin and the undetectable level of GFAP in BMSSCs (BM). GAPDH, a housekeeper gene, served as a PCR amplification control. M = markers.

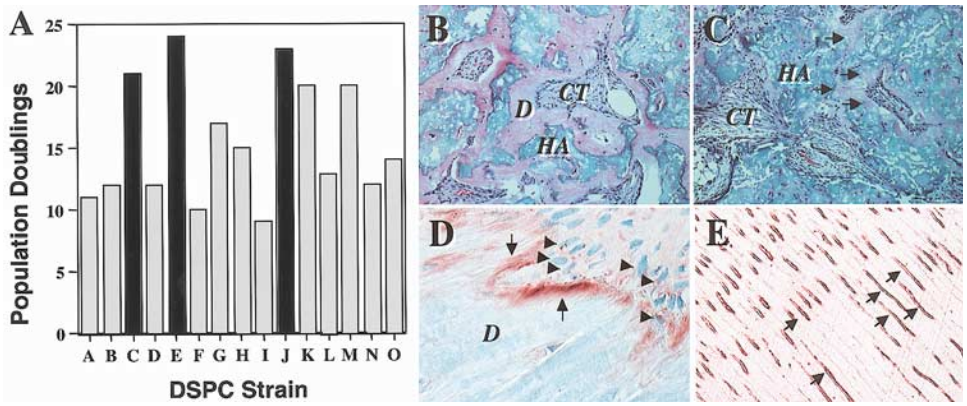

Figure 4. Cloning efficiency of DPSCs. **(A)** Of 15 selected single-colony-derived strains of DPSCs, only 3 were capable of proliferating over 20 population doublings (PD) (strains #C, #E, and #J). These 3 strains proliferated beyond 20 PDs (final PD is not yet identified), but all other strains proliferated only between 10 and 20 PD. These strains were not able to generate enough cells for study of their developmental potential. Altogether, 12 single-colony-derived DPSC strains (including strains #C, #E, and #J presented here), capable of exceeding 20 PD, were developed and tested with respect to their ability to form a dentin-pulp-like complex *in vivo*. After approximately 25 PDs, they were transplanted subcutaneously into immunocompromised mice for 8 wks. Two-thirds (8 of 12) of these single-colony-derived DPSC strains formed the same amount of dentin as multi-colony DPSCs **(B)** and 1/3 (4 of 12) generated only a limited amount of dentin **(C)**. Newly formed dentin (arrows) and odontoblasts (triangles) were immunoreactive for DSP antibody in single-colony-derived DPSC transplants **(D)**. Positive control, demineralized human dentin section, showing the peritubular areas immunostained with human DSP antibody (E, arrows).

antibodies against a cell-surface marker CD29. The majority of the isolated stromal-like cells were further shown to be positive for a human-specific alu DNA probe, with a minor population of cells expressing the mouse-specific pf1 DNA probe by *in situ* hybridization (Figs. 2C, 2D). After expansion *in vitro*, human cells were re-transplanted into immunocompromised mice. The secondary transplants yielded human alu-positive odontoblasts that gave rise to a dentin-pulp-like complex containing organized collagen fibers detected by Trichrome staining (Figs. 2E, 2F). Importantly, the regenerated dentin was immunoreactive for human DSP antibody (Fig. 2G). These findings indicated that human DPSCs satisfy one important stem cell attribute in their ability to self-renew *in vivo*.

We studied the potential of DPSCs to differentiate into adipocytes and neural cells, in analogy to what has been demonstrated in BMSSCs. After 5 wks of culture with an adipogenic-inductive cocktail, Oil red O-positive lipid clusters were identified in DPSC cultures (Fig. 3A). This correlated with an up-regulation in the expression of two adipocyte-specific transcripts, PPARγ2 and lipoprotein lipase, detected by RT-PCR (Fig. 3B). Furthermore, DPSCs were found to express nestin and glial fibrillary acid protein (GFAP), markers of neural precursors and glial cells, respectively, at both the mRNA and protein levels (Figs. 3C-3G). These results suggested that DPSCs are similar to other stem cell populations, such as BMSSCs, in possessing the ability to develop into developmentally diverse phenotypes.

We next examined the characteristics of strains that originated from a single cell. The majority of the progeny (80%) derived from these single colonies proliferated for less than 20 population doublings (Fig. 4A). This indicates that the majority of clonogenic DPSCs do not grow extensively *ex vivo* and that only a small percentage of clonogenic DPSCs (20%) will ultimately represent the cell population at later PDs. Interestingly, when 12 single-colony-derived DPSC strains with the ability to proliferate over 20 population doublings were transplanted into immunocompromised mice, the majority (two-thirds) demonstrated a potential equivalent to that of multi-colony-derived DPSCs in being able to generate abundant ectopic dentin on and in the HA/TCP carrier (Fig. 4B). However, the remaining third of the single-colony-derived DPSC strains formed only a moderate to low amount of dentin when compared with multi-colony-derived DPSCs (Fig. 4C). The dentin-pulp-like complex formed by each individual colony, however, demonstrated the same structural properties as its parental clone. The regenerated dentin and normal dentin were positive for human DSP, as shown by immunohistochemical staining (Figs. 4D, 4E). Under the same immunohistochemical staining conditions, BMSSC transplants were negative for human DSP staining (data not shown). These results imply that DPSCs may contain subpopulations of cells with different proliferative rates and developmental potentials, a property similar to that of BMSSCs. Human DPSCs were also identified in the pulp-like connective tissue, even 5 mos post-transplantation (Appendix Fig., www.dentalresearch.org), indicating that this population of cells might be the ones responsible for the self-renewal.

DISCUSSION

These results show that human DPSCs are capable of self-renewal following *in vivo* transplantation. Theoretically, DPSCs are capable of responding to specific environmental signals and either to generate new stem cells or to select a particular differentiation program. Our observations have provided preliminary evidence suggesting that transplanted DPSCs can not only commit to the odontoblast lineage but also reside in the pulp-like connective tissue as fibroblast-like cells, even at 5 mos post-transplantation (Appendix Fig., www.dentalresearch.org). It is possible that these fibroblast-like cells belong to a population of more primitive reserve cells responsible for the dentin formation in the secondary transplantation.

Recently, it was reported that DSPP may also be expressed in bone, albeit at low levels. In the present study, we found that an antibody specific to DSP protein localized to the peritubular dentin area and to the odontoblast layer in sections of human teeth by immunohistochemical staining. This DSP antibody may be not sensitive enough to detect DSP antigen in BMSSC transplants, if in fact there is any.

Based on current information, we suggest that DPSCs may have a broader capacity for differentiation than originally thought. Although it seems probable that several different cell types reside in pulp tissue, adipocytes are not a normal cellular component in dental pulp. We initially reported that DPSCs were unable to develop adipocytes following treatment with the glucocorticoid, dexamethasone, as is seen in dexamethasone-induced BMSSCs

(Gronthos *et al*., 2000). However, we now report that a more potent adipogenenic-inductive culture medium (Gimble *et al.,* 1995) can induce DPSCs to form characteristic oil red O-positive lipid-containing adipocytes. This phenotypic conversion was also correlated with the expression of the early adipogenic master gene PPARγ2 and the late marker lipoprotein lipase. Recently, neuronal stem cells were reported to be isolated from dermis, a tissue that contains abundant nerve fibers (Toma *et al*., 2001). Dental pulp also contains prominent nerve fibers, which penetrate the dentin tubules. Previous reports provided evidence that nestin and GFAP could be detected in pulp cells (Davidson, 1994; About *et al*., 2000), and pulp cells might even be capable of producing a variety of neurotrophins (Nosrat *et al*., 2001).

Analysis of dentin formation *in vivo* by single-colony-derived strains of human DPSCs showed that most of the colonies (80%) failed to proliferate beyond 20 population doublings (PD). Thus, these strains cannot be expanded *ex vivo* to produce sufficient numbers of cells to analyze all their developmental potentials *in vivo*. We therefore utilized those single-colony-derived DPSC strains which had the potential to proliferate at least over 20 PD. Multi-colony-derived DPSCs of 20 to 30 PD were consistent in their capacity to proliferate *in vitro* and to regenerate dentin *in vivo*. Based on our results, only 67% (8 out of 12) of the highly proliferative single-colony-derived DPSC strains were capable of forming the abundant amounts of dentin comparable with the parental multi-colony-derived cultures. Analysis of the dentin matrix formed by single-colony-derived strains demonstrated a mineralized dentin matrix, containing organized collagen fibers, similar to that formed by multi-colony-derived DPSCs. Collectively, these studies suggest a hierarchy of progenitors in adult dental pulp, including a minor population of self-renewing, highly proliferative, multi-potent stem cells, among a larger compartment of perhaps more committed progenitors. The concept of a hierarchy of cellular differentiation has previously been described for other stem cell populations, such as BMSSCs (Kuznetsov *et al*., 1997). In conclusion, we provide compelling evidence to show that DSPCs belong to a novel population of post-natal somatic stem cells. These cells can serve as a model for the study of adult stem cell differentiation *in vitro* and tissue regeneration *in vivo*.

ACKNOWLEDGMENTS

This work was supported by the Division of Intramural Research, the National Institute of Dental and Craniofacial Research, the National Institutes of Health.

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